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锯缘青蟹 *Scylla serrata*(Forskål)胚胎发育的基础研究

Foundational studies on the embryonic development of the mud crab, *Scylla serrata*(Forskål)

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摘 要

锯缘青蟹 *Scylla serrata*(Forskål，1775)是我国重要的养殖经济蟹类，胚胎发育在个体发生中处于重要地位。本实验采用组织学、光学显微镜、电子显微镜观察以及离体培养等技术与方法，对锯缘青蟹胚胎发育过程进行了系统的研究，主要结果如下：

1 锯缘青蟹排卵大多发生在晚 12:00 前后，成熟卵子入水后，无论受精与否，最外层卵黄膜都会举起形成壳膜，随即壳膜形态结构和性质发生变化，其外层膜不连续的地方形成微孔，而内层膜去致密化的絮状粘稠物形成粘胶，粘胶通过微孔渗出到卵子表面，壳膜变得富有粘性、延展性和可塑性。在亲体腹肢不断摆动和水流的作用下，壳膜或其上的皱褶相互接触或接触到刚毛上而分别形成卵索和卵柄，卵索和卵柄与壳膜是连续一体的，并逐渐固化变得坚硬和韧性，最终卵子通过卵索和卵柄而牢固附着在亲体的腹部。本实验首次在十足目甲壳动物卵子中发现微孔和粘胶，证实了卵子粘性来自于卵子本身；从而提出卵子附着的壳膜粘连固化的学说。

2 锯缘青蟹精子入卵后，发生皮层反应而形成受精膜。皮层反应包括致密颗粒首先胞吐以及环形颗粒(Rg)的相继多轮胞吐。每轮 Rg 的胞吐可以分为受精膜(Fm)的平缓期、形成期和举起期三个阶段。Rg 的形成与卵黄颗粒和脂滴以及线粒体密切相关。本实验首次提出线粒体的形态、数量及分布可以很好反映 Fm 形成的不同阶段，并在国内首次报道 Rg 的发生以及其胞吐的全过程。

3 锯缘青蟹成熟卵子为初级卵母细胞，核相处于第一次成熟分裂的中期。在卵子产出 30min 内第一极体排出并分裂成两个小极体;第二极体随着 Fm 的形成和举起而排放,在卵子产出后 50-60min 内排出;第一,二极体分别位于 Fm 的内外两侧。雄性原核形成早于雌性原核;雌雄原核形成后向卵子中央移动,最后发生联合形成联合核。排卵后的 6-8hr 内,合子核才开始第一次核分裂。锯缘青蟹为数精入卵，单精受精。

4 锯缘青蟹的胚胎发育分为 10 期：Ⅰ受精卵、Ⅱ卵裂期、Ⅲ囊胚期、Ⅳ原肠期、Ⅴ无节幼体期、Ⅵ五对附肢期、Ⅶ七对附肢期、Ⅷ复眼色素形成期、Ⅸ近孵化期、Ⅹ幼体孵出。对 10 期形态特征进行细致描述并编制了锯缘青蟹胚胎发育表，为育苗生产中胚胎发育的检测提供指导。

5 锯缘青蟹胚胎原肠期之前，卵黄颗粒与脂滴呈网状分布；从原肠期至五对附肢期卵黄颗粒开始裂解成卵黄絮状物质，脂滴开始溶解而愈合和吸收；七对附肢期已见不到完整的卵黄颗粒，卵黄和脂滴正在快速溶解和吸收；到复眼色素形成期卵黄絮状物质更加稀疏，偶尔见到脂滴残余。青蟹卵黄和脂滴的溶解与吸收是胚胎四周先于胚胎中央。胚胎四周溶解的卵黄絮状物质和脂滴迅速扩散，参与细胞的分裂和分化以及膜状结构的合成而被吸收；胚胎中央的卵黄絮状物质较致密大团，大脂滴团迅速溶解消失于絮状物质中，并参与形成卵黄致密颗粒，这些致密颗粒随同絮状物质扩散。胚胎中央卵黄絮状物质中出现髓样小体、线粒体、核糖体、细胞核和内质网等结构。本文首次报道甲壳动物胚胎发育过程中卵黄和脂滴的消化吸收情况，认为卵黄和脂滴为胚胎提供营养物质和膜原料，它们的消化吸收与胚胎器官和系统的快速形成和发育相关。

6 根据外部形态特征，锯缘青蟹胚胎复眼发生可以分为 4 个阶段：1.复眼色素带(Eps)出现期：视叶外侧出现 1 对桔红色丝状 Eps；2.Eps 斑状期：Eps 增大为小椭圆形斑状，呈棕红色；3.Eps 条状期：Eps 加长加粗为长条形，呈深棕红色，小眼分界开始出现；4.Eps 核仁期：Eps 迅速加深并扩张为大椭圆球状，呈棕黑色，小眼界限分明呈放射状。此外，孵出幼体的黑色半球状复眼着生在粗短的眼柄上，由许多六边形的小眼组成。

超微结构显示：锯缘青蟹复眼内小眼的感光部分在胚胎阶段由 7 个小网膜细胞组成，第一期蚤状幼体由 11 个小网膜细胞组成；感杆束由每个小眼中的小网膜细胞伸出的微绒毛相互交汇形成的，在小眼中央呈椭圆形，细胞核分布于小网膜细胞的远端；随着小眼的发生，感杆束和色素颗粒不断形成和增大，各种细胞器增多；复眼的感光功能是逐渐完善的。

7 锯缘青蟹胚胎体色素发生与复眼发生关系密切：Eps 出现期，体色素未出现；Eps 斑状期，开始出现小圆形色素斑，并逐渐形成复眼体色素带、心脏体色素带和腹部体色素带，3 对体色素带彼此相互接连；Eps 条状期，色素斑扩大呈星芒状，体色素带变粗变深，新色素斑在形成；Eps 核仁期，色素斑呈树枝状延展，体色素带扩大变为浓厚色素团，心脏体色素带最为显著。本实验首次系统研究了胚胎体色素的发生过程，并提出色素细胞的分布和颜色变化可以作为胚胎发育阶段的一个重要参考指标。

8 锯缘青蟹抱卵孵化、剥离受精卵和“流产”卵离体培养的孵化率分别为 90-100%、30-50%、20-50%，说明剥离卵的离体培养在生产上意义不大，而“流产”卵有一定的应用价值。锯缘青蟹胚胎原肠期是离体培养的关键时期，过了原肠期的附着卵胚胎离体培养的孵化率可以提高到 80％以上。对锯缘青蟹胚胎在大中小水体，不同处理方式，以及不同类型胚胎的离体培养进行了比较研究，并提出了小水体的“分离培养”这一很有价值的模式。锯缘青蟹胚胎发育的异常现象常有微生物感染、异常发育和不同步发育等三种情形。

9 借助光镜和电镜观察基本弄清锯缘青蟹胚胎膜的层数和发生时序：成熟卵子入水受精而形成的壳膜（EE1）和 Fm（EE2）构成孵化膜(Hm)，之后胚胎相继分泌形成 EE3、EE4 和 EE5，青蟹的胚胎膜总共有 5 层。我们将胚胎膜定义为除表皮以外胚胎表层具有的明显分层的膜状结构，并对胚胎膜的生物学意义进行论述。锯缘青蟹各胚胎膜的来源和特性仍待深入研究。

10 锯缘青蟹幼体的抱卵孵化一般在凌晨 4:00-8:00，亲体腹肢扇动对幼体的成功孵出以及同步孵化都相当重要。离体培养胚胎的幼体孵化在一天各个时段都有出现，没有呈现幼体孵化时间的节律性和同步性。青蟹幼体正常孵化时，内层胚胎膜先于外层孵化膜破裂;非正常孵化时，胚胎膜破裂顺序则表现为由外至内:壳膜、Fm、EE5、EE4。

关键词： 锯缘青蟹； 胚胎发育；胚胎膜；复眼；色素带；离体培养

Abstract

Scylla serrata(Forskål，1775) is an important econimic aquatic breeding crab. Embryo takes a significant position in individual ontogenesis.With the histological techniques,optical microscopy,electron microscopy，*in vitro* culture techniques,a systematical and fundamental study was made on the embryonic development of *Scylla serrata*.The main results as follows:

1.The female crabs of *Scylla serrata* usually ovulated during the time of evening to dawn.As the mature oocytes passed through the gonopore and immerged into sea water,whether fertilized or not, their vitelline membranes swelled and formed chorin, immediately the structure and character of chorin changed greatly:with the considerably swelling of chorin， micropores appeared at the incontinuous exochorin;while some of glue,which were produced by endochorin disadensifying flocculent materials, penetrated through the micropores and attached chorin，so chorin became sticky,pliable and extended.With the rapid abdominal pumping of the ovigerous females and the water flowing,a portion of chorin became deflected off the egg's surface,then chorin and its wrinkles were entangled with each other or caught by ovigerous setae and formed funiculuses and egg stalks respectively.Funiculuses and egg stalks mentioned here，were the different extensional part of chorin，which finally condensed and concreted to form a tough and durable material capable of anchoring the egg mass to the pleopods.We firstly demonstrated the existence of micropores and glue of oocyte in crustaceans, which approved the sticky glue for attachment was from the eggs themselves, and we developed chorin conglutinated-condensed-solidified theory of egg attachment.

2.The second envelope,the fertilization membrane(Fm)，formed between oocyte and chorin during a complex cortical reation initiated after fertilization.The cortical reation includes the successive exocytosis of two morphologically different granules.Firstly the dense granules undergone exocytosis and formed a thin membrane.As followed that,the ring-shaped granules undergone several rounds of massive and rapid exocytosis,the material from them fused and formed a thick membrane.Every round of exocytosis of the ring-shaped granules were divided into three stages as the placid，the forming and the swelling of Fm.The development of the ring-shaped granules were closesly connected with two kinds of yolk granules and lipid drops and mitochondrias.Finally，the fertilization membrane was composed of the two layers,and the newly formed plasma membrane became an inlaying membrane(the third envelope).It is the first time reported the development of the ring-shaped granules and its exocytosis process in China and mentioned that the quality and distribution of mitochondria could reflect the different stages of the fertilization membrane developing.

3.The ripe egg was the primary oocyte that was at meiotic metaphase Ⅰ stage. The meiotic apparatus positioned perpendicularly to the egg surface. The chromosomes drawn by the spindle microtubule separated. The chromatin near the plasma membrane was surrounded and formed the first polar body. After fertilization membrane was lifted, the secondary oocyte emitted the second polar body into the perivitelline space.The first and the second polar bodies were extruded before and after the formation of fertilization membrane,within 30min and 60min after fertilization and located outside and inside of the fertilization membrane respectively. The sperm nucleus and the monoploid female nucleus became the male and the female pronucleus respectively, and the male pronucleus formed earlier than the female’s. The male and the female pronucleus moved closer toward each other and associated to form a association nucleus in the center of the egg. The chromosomes of the first mitotic apparatus separated 6~8hrs after fertilization and 2-cell embryo was formed by plasma membrane invagination. In *Scylla serrata*, several sperms penetrated into the egg, and only one sperm fertilized the egg.

4.The embryonic development in *Scylla serrata* was divided into 10 stages: StageI-fertilized egg, StageII-cleavage, StageIII-blastula, StageIV-gastrula, StageV-nauplius, StageVI-5 pairs of appendages, StageVII-7 pairs of appendages, StageVIII-eye-pigment formation, StageIX-prehatching, StageX-hatching.The morphological character of 10 stages was particularly described and a embryonic development timetable in *Scylla serrata* was editted, and it would provided inspection and forecast for the embryonic development in the breeding activity.

5.Yolk granules and lipid drops were reticulated before StageIV(gastrula) in *Scylla serrata* embryo.From StageIV to StageVI (5 pairs of appendages), yolk granules began to split into flocculent material，which dispersed around;while lipid drops dissolved and fused，then melted and absorbed.No whole yolk granules were found at the StageVII (7 pairs of appendages), yolk and lipid were melted and absorbed quickly.At the StageVIII (eye-pigment formation), yolk flocculent material was more sparse, lipid remnants were casually visible.The dissolving and absorbing of yolk and lipid happened earlier at the embryo arounding than in the embryo center.The dissolved yolk and lipid at the embryo arounding were expanded quickly and absorbed to attend the cell differentiation and the forming of membrane structure.While in the embryo center,large large lipid drops, rapidly dissolved and dispersed into the yolk flocculent material which is much denser and larger,attended to form yolk dense granules,and they expanded with the yolk flocculent material,in which myeloid body, mitochondria, ribosome, nucleus, endoplasmic reticulum and other organelles appeared. It is reported for the first time the yolk and lipid absorbing status during embryogenesis in crustaceans, which provide nutrition for embryo cell and material for membrane，as well as their dissolving and absorbing are connected with the development of embryonic organs and systems.

6.The development of the compound eyes in *Scylla serrata* embryo could be divided into 4 phases according to morphological characters:VIII1-Eps appear phase:a pair of orange thin riband lacy Eps appeared near the outiside of optic lobe. VIII2-Eps speck phase:the Eps enlarged to be red small ellipse. VIII3-Eps oblong phase:the Eps lengthened to be brown oblong,the dividing lines of ommatidia appeared. VIII4-Eps ellipsoid phase: the Eps rapidly widened and fully developed to be black large globosity, the limit of ommatidia became clear and radiate dition. In addition, the dark half-ball-like compound eye of Zoea I, which fixed at the stubby eyestalk,was composed by plenty of hexagon-like ommatidia.

The ultra-structure of compound eyes showed that: The sensate part of an ommatidium was composed by 7 retinular cells(RCs) in embryo,and 11 RCs in Zoea

I.The rhabdomer was composed of the interlaced microvilli which stretched out from the RCs and located in the center of an ommatidium like ellipse.Nucleus was in far beyond the RC.With the development of ommatidia,the rhabdomers and pigment granules were forming and enlarging ceaselessly,and various of organelle were mutiplied.The fine structure indicated that the function of the light sensation in Scylla serrata was gradually consummated.

It is the first time that Eps was adopted as the criterion of stages both for the compound eyes'development and for the embryonic development in Brachyura embryo.

7.The development of somatic pigment was tied up with the development of Eps in the embryo of *Scylla serrata.* VIII1-Eps appear phase: chromatophores(Cps) not appeared yet. VIII2-Eps speck phase: small round Cps appeared and formed eye somatic pigment bands(Espbs),heart somatic pigment bands(HSpbs) and abdomen somatic pigment bands(ASpbs). These 3 pairs of Spbs were interactive.VIII3-Eps eblong phase: Cps expanded as radiant. Spbs broadened and darkened.New Cps were developing. VIII4-Eps ellipsoid phase: Cps expanded as dendriform. Spbs enlarged as dark mass,in which HSpbs were the most remarkable. It is the first time in our experiment the development of embryonic somatic pigment and point out that the distributing of Cps and its color changing can be an important index for embryogensis.

8.In *Scylla serrata*，the hatching rate of AE incubated by female、PE and AbE under *in vitro* culture was 90-100%、30-50%、20-50% respectively.The data showed that *in vitro* culture of PE had less value for mass production compared with AbE. StageⅣ gastrula in the embryo of *Scylla serrata* was a significant stage in IVCEb since the hatching rate of AE embryo after gastrula under IVC could be promoted higher than 80％.We had a parallel study for different types of embryo,with different dealing methods, under different breeding environment(as in LV,MV,SV) and found a valuable mode for IVCEb,which was called "separate breeding in SV" or "mini-culture".There were three abnormal phenomenon during embryonic development in *Scylla serrata*: the infection of microbes, abnormal development, develop non-synchronizely.

9.The layers and its sequence of the embryonic coat(EC) in *Scylla serrata* were certified with optical microscopical, electron microscopical observations .After the ripe eggs immerged into water and fertilized, it developed EE1(Ch) and EE2(Fm)，which constituted hatching membrane(Hm).As followed that,the embryo continually developed EE3, EE4, EE5.There were altogether 5 envelopes of EC in *Scylla serrata*.We defined EE as the clear layer structure around embryo except the cuticle and its biological significance of the EC was also discussed.But the source and character of the EEs in *Scylla serrata* still need further study.

10.The larval hatching time in *Scylla serrata* is usually at 4:00-8:00 a.m.. The rapid fanning behavior of the female crab was very important for the successfully hatching and synchronizing hatching.The embryo under *in vitro* culture lost the hatching rhythm and synchronization. The normal larval hatching shows that the breaking of inner embryonic envelopements was ahead of the outer. However, the sequence of the abnormal hatching was the opposite:EE1,EE2,EE5,EE4.

Key Words: *Scylla serrata;* Embyonic development; Embyonic envelope; Compound eyes; Pigment band; *In vitro* culture

第一章 绪论

第一节 十足目甲壳动物胚胎发育的研究进展

1.1 前言

动物胚胎学是一门古老而又热门的学科。1995 年果蝇胚胎发育过程中基因调控的研究获得诺贝尔生理和医学奖，划出了胚胎发育研究的又一个黄金时代。现在正在运用遗传学、生物化学和分子生物学手段进行深入探讨人类和最重要模式生物发育机制。

胚胎发育指在一稳定的遗传信息库中基因经选择性的表达，由单细胞受精卵产生一个复杂成体的过程。胚胎发育的起点是受精的卵细胞，它能分裂增殖，并按照一定的时空顺序沿着模式形成进行细胞分化和迁移，形成各型细胞和超细胞结构（组织，器官），最终成为有机体。

轴和极性以及核质关系是胚胎发育上的两个难题。而生物模式形成是发育生物学的中心问题，模式形成是关于细胞在空间上有次序地分化，从而引起结构有序排列。模式成因在于基因水平的重组与细胞的社会性两方面。

胚胎学的发展一直是依赖于少数模式生物取得的惊人进展。集中与积累的结果使得对胚胎发育的基础过程的研究日趋完善，并上升到分子水平，且易取得实质性的突破。现已从分子水平深入探究胚胎发育中组织分化、器官形成和胚胎定形的机理。而最近几年研究工作者重返和深化传统研究，其主要目标是胞质决定子与 DNA 的相互作用及细胞间的信号交换[176]。

甲壳动物胚胎学的研究始于 19 世纪早期，真正广泛的研究则从 19 世纪后半叶起。胚胎发育的研究离不开实验技术和方法的发展。1920 年之前，实验技术的不完善（Reichenbach 1888，用徒手切片法研究甲壳动物胚胎发育），在研究对象和内容上就受到限制。集中在十足目中含有大型卵的种类[108]。1920-1945 年，组织切片技术的引入，使研究进入发展阶段。1945 后的很长时间实验技术上没有多大的突破，只是对传统实验方法的改进和完善。到 1987 年才有学者将扫描电镜技术（Celade,1987,1991）、1992 年将生化技术[64]引进甲壳动物胚胎学的研究中来。近十几年来，才开始逐渐将在昆虫胚胎发育研究中应用的传统遗传学和在脊椎动物的胚胎发育研究中应用的化学方法、透射电镜技术以及在这两类动物胚胎学研究中广泛应用的分子生物学方法逐渐地应用到甲壳动物胚胎学的研究

领域中来。兹结合近些年来国内外在十足目经济甲壳动物胚胎上的研究，尤其在虾蟹类上做的一些工作进行一些总结。

从动物整个发育过程来看，个体发育包括了胚前期、胚胎期和胚后期。胚前期是指生殖细胞在亲体内的发生。胚后期一般指幼虫或幼体自脱离卵膜或母体起到性成熟为止，但广义上包括了成年期和衰老期。而胚胎期是指自受精卵开始卵裂到胚胎脱离卵膜或母体为止，这时期的发育是依靠卵子内贮存的卵黄或直接由母体输送的养料而在卵膜或母体内进行，动物的部分成体器官和某些幼虫特有的器官都是在此时期里开始发生和基本形成的。可见胚胎作为亲体和幼体的中间环节，处在举足轻重的位置；胚胎发育是个体发育的主体，一直是发育生物学研究的核心和前沿。尤其是近些年来，遗传学、生物化学和分子生物学等手段正被广泛运用在人类和重要模式动物发育机制的研究上，同时越来越多地借助于计算机模型技术来解决生物模式形成等复杂问题。甲壳纲是节肢动物门的大纲，然而甲壳动物胚胎学的研究却开始于 19 世纪早期[86]，虽经过一个多世纪的发展，但研究的资料多集中在形态发生方面，有关胚胎生理、生化和遗传等内容很少。所以今后需要运用当前的各种先进科学技术手段，更深入地研究甲壳动物胚胎的发育机理和调控机制，以便促进甲壳动物胚胎学的发展，更好地推动生产实践。

离体培养或称体外培养(*in vitro* culture，IVC)就是将活体结构成分（诸如活体组织、活体细胞或者活体器官等)甚或活的个体从体内或其寄生体内取出，放在类似于体内生存环境的体外环境中，让其生长和发育的方法。按照体外培养的结构成分，可以分为组织培养（tissue culture)、细胞培养（cell culture）及器官培养(organ culture)等类别[87]。传统上，体外培养是相对体内培养（*in vivo* culture）而言的。

胚胎的离体培养（*in vitro* culture of embryo，IVCEm）就是指将胚胎从亲体体内取出或从亲体身上取下，放置于类似于胚胎自然生存环境的外环境中，让胚胎生长和发育的方法。传统意义上的胚胎离体培养通常是在卵胎生的动物中进行的，将胚胎从体内取出放置于胚胎培养液中进行培养；而且是与体外受精(*in vitro* fertilation，IVF)密切相关的重要技术。IVCEm 大量的工作集中在家畜等哺乳类动物，已经是相当成熟而被广泛应用的技术。然而十足目动物的胚胎离体培养是另一种意义上的离体培养。因为十足目除对虾科的受精卵直接散落水中

外，其余所有种类的雌体则都用腹肢抱卵，受精卵固着在腹肢的刚毛上，直到孵化[20]。十足目动物的胚胎离体培养是相对于绝大多数抱卵孵化的动物而言的，就是让胚胎脱离亲体进行孵育培养，是相对亲体抱卵孵育而言的；当然也包括了体外受精后的受精卵胚胎的离体培养。所以，十足目动物的胚胎离体培养一般分为人工受精卵胚胎离体培养和自然受精卵胚胎离体培养。而后者又可以分为附着卵胚胎离体培养和“流产”卵胚胎离体培养。

离体培养技术是于 19 世纪后叶伴随着实验胚胎学的迅速兴起而拉开序幕的[87]。

1.2 壳膜和卵柄的来源及卵子的附着机制

十足目甲壳动物一般存在内部型纳精囊和外部型纳精囊两种类型。前种类型的物种的亲体卵巢的卵子经过纳精囊进行体内受精后从生殖孔排出体外到刚毛丛和水体中,而后被载卵刚毛附着。而后种类型亲体的成熟卵子从生殖孔被挤出先经体外纳精囊进行体外受精后,继续沿着腹部表面移向腹肢形成的孵育室内的粘液中驻留一段时间才被载卵刚毛附着[124]。抱卵现象在十足目甲壳动物中十分普遍,除对虾科外,其余所有种类的卵子排出体外后都依靠卵膜与腹部内肢载卵刚毛所形成的卵柄以及卵与卵之间的卵膜的延展形成的卵索而附着于雌体腹肢上[20]。成功的卵子附着对卵的保护和正常发育以及幼体的散布具有重要的生物学意义。因此,一个多世纪以来,许多学者对十足目甲壳动物不同种类的卵子附着机制展开了广泛深入的研究[21, 36, 84, 89, 106, 117, 119, 122, 124, 143, 149, 150, 151, 153, 154, 188, 189, 191, 201, 202, 205, 207, 209, 214, 216, 219,],并提出了相关的附着机制的理论和假设。然而不同的学者提出的观点存在很大的矛盾和争议。其中外层卵膜和卵柄的来源以及卵子通过卵柄以何种方式附着于载卵刚毛上是整个争论的焦点。但是众说纷纭,在这两个经典问题上至今仍未得到圆满的结论。

自 Braun[116, 117]首次发现并描述了十足目甲壳动物的粘液腺以来,有关外层卵膜和卵柄的来源问题的争论主要围绕粘液腺的有无以及粘液腺在卵膜和卵柄形成中的作用而展开。早期学者普遍认为甲壳动物不仅有粘液腺,而且是粘液腺分泌的粘液形成外层卵膜和卵柄。Braun[117]、Broekhuysen[119]， Yonge[216, 219]、Stephens[202]、Aiken & Waddy[106]分别观察了刺蛄属(*Astacus*)、滨蟹(*Carcinides maenas*)、普通海刺蛄(*Homarus vulgaris*)、普通褐虾(*Crangon vulgaris*)、美